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SHORT COMMUNICATION

THE PHOSPHORYLATION OF GERANIOL IN GERMINATING PEAS*

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Abstract—Geraniol-1-^{3}H and nerol-1-^{3}H were prepared from their corresponding aldehydes. The labelled geraniol led to labelled squalene in a homogenate of germinating peas and to a labelled acidic ester. Since hydrolysis with alkaline phosphatase liberated geraniol, this ester is assumed to be geranyl pyrophosphate.

INTRODUCTION

THAT the pyrophosphates of isopentenoid alcohols are involved in stepwise polymerization to give compounds such as farnesyl pyrophosphate is well established.[1–4] The available evidence on the presence of mevalonic acid kinase suggests that phosphorylation normally occurs at the mevalonate stage.[5–12] Furthermore, it is known[13,14] that *Pseudomonas citronellolis* degrades the free isopentenoid alcohols (citronellol, geraniol, and farnesol) to acetate. On the other hand, a geraniol kinase has been reported[15] and free isopentenoid alcohols have been shown to proceed to β-carotene[16,17] and to squalene and its metabolites.[18] In the latter case label was incorporated from geraniol-^{14}C in germinating peas. We would like to present evidence which indicates that the pea phosphorylates geraniol.

RESULTS

Geraniol labelled with tritium at C-1 was prepared by the reduction of the corresponding aldehyde with sodium borotritide. When it was incubated with a homogenate of peas,

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squalene was obtained in a 0·3–1·8 per cent yield depending on the amount of geraniol incubated. The hydrocarbon was isolated as the hexahydrochloride of m.p. 109–112° recrystallized to constant specific activity (Table 1). The retention of radioactivity (40 per cent) in the 109–112° isomer compares favorably with values reported earlier.[19]

TABLE 1. COCRYSTALLIZATION OF SQUALENE HEXAHYDROCHLORIDE WITH HEXAHYDROCHLORIDE OF METABOLITE FROM GERANIOL-1-^{3}H

Crystallization no.	Specific activity (dpm/mg × 10^{-4})	m.p. (°)
0	7·98	
1	3·21	107–111
2	3·06	108–110
3	3·15	109–112
4	3·22	109–112
5	3·17	109–112

The specific activity at crystallization No. 0 is the radioactivity in the hydrocarbon fraction from alumina chromatography divided by the weight of added carrier squalene hexahydrochloride.

A similar incubation was carried out with geraniol-1-^{3}H except that the homogenate was well extracted with ether to remove squalene and unchanged geraniol and then extracted with collidine which extracts pyrophosphates.[4] The collidine extract contained 1·9 per cent of the radioactivity of the original substrate. The material in the collidine was transferred to an aqueous solution which was again extracted with ether to remove traces of geraniol and squalene. The aqueous layer was then hydrolyzed with calf intestine alkaline phosphatase. The hydrolysis product was examined by gas–liquid chromatography with simultaneous measurement of radioactivity in the effluent (Fig. 1) and the liberated alcohol was found to

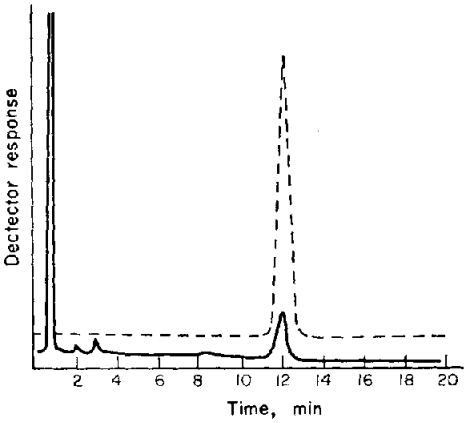

FIG. 1. ISOTHERMAL (185°) GAS-LIQUID CHROMATOGRAPHY OF THE GERANIOL-1-^{3}H METABOLITE WHICH WAS SOLUBLE IN COLLIDINE AND RELEASED BY ENZYMATIC HYDROLYSIS. SOLID LINE IS MASS AND INTERRUPTED LINE IS RADIOACTIVITY. AN ALIQUOT EQUIVALENT TO 3·0 × 10^3 dpm WAS INJECTED. AUTHENTIC GERANIOL WAS ADDED AS CARRIER AND APPEARS AS THE MASS PEAK AT 12 min. NEROL WOULD HAVE APPEARED ABOUT 2 min EARLIER. THE MASS PEAK BELOW 1·0 min IS SOLVENT.

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be geraniol. No other radioactive product was observed from hydrolysis. The GLC technique was sufficiently sensitive to distinguish as small a change as *cis–trans*-isomerism as described more thoroughly in the experimental section.

DISCUSSION

The conversion of geraniol-1-^{3}H to a collidine-soluble product which released labelled geraniol on enzymatic hydrolysis can only mean that a polar ester was formed. Since geraniol-1-^{3}H was also converted to squalene under the same conditions with a pea homogenate and since geraniol-1-^{14}C has been converted[18] to squalene in the pea under *in vivo* conditions, it is reasonable to assume that the ester formed was a phosphate, presumably a pyrophosphate. The existence of a kinase for geraniol therefore seems reasonably certain in the pea. Whether it is specific for geraniol or whether it is, for instance, the same kinase which phosphorylates mevalonic acid remains to be demonstrated.

The metabolic utilization of the free isopentenoid alcohols may open the way for easier experimentation of the pathway by which many terpenoids are formed, since the alcohols are preparatively easier to obtain than the phosphorylated derivatives. We report in this paper a simple method by which both geraniol-1-^{3}H and its *cis*-isomer, nerol-1-^{3}H, can be made. Commercial citral was found to be a mixture of geranial and neral which on reduction with tritiated sodium borohydride yielded a mixture of the C-1-tritiated alcohols. The latter were then separable by preparative gas–liquid chromatography.

EXPERIMENTAL

Methods

Radioactive counting was performed on a Nuclear-Chicago automatic scintillator or planchette counter. Gas–liquid chromatography was performed on an F and M Model 400 instrument equipped with a 3·8 m × 6 mm copper tube packed with 15 per cent carbowax on Chromosorb-W (60–80 mesh). The effluent was split so that 10 per cent passed into a flame detector and 90 per cent into a Nuclear-Chicago proportional counter.

Materials

Citral (Florasynth Laboratories) was found by GLC to be a mixture of 58 per cent geranial and 42 per cent neral. It (98 mg) was reduced with 6·08 mg of sodium borotritide (New England Nuclear Corp. 200 mc/mM) in 300 μl of isopropyl alcohol. The reaction mixture was warmed to the boiling temperature and then allowed to cool for 15 min. The mixture was diluted with 600 μl of ether and washed twice with water. The products were separated by preparative GLC and collected in glass tubes equipped with an electrostatic precipitator[20] (without the precipitator the alcohols remained as a fog and could not be collected). The precipitator did not affect the structures of the alcohols. The collected geraniol and nerol from a larger nonradioactive preparation had the same i.r. spectra as authentic geraniol ("Meranol", Compagnie Parento, Inc., 99 per cent pure by our GLC analysis) and nerol (Fluka, AG., 97 per cent pure by our GLC analysis). They were distinguishable in the 9 μ region where nerol has a doublet with 0·20 μ separation of the peaks and geraniol has a doublet with 0·15 μ separation. In addition the GLC retention times of the two synthetic alcohols were the same as for the authentic samples. Nerol appeared earlier and was completely separated from geraniol. Under programmed temperature conditions (5°/min from 100° to 200° with program delay of 6 min at 100°) nerol had a retention time (relative to xylene) of 2·87 and geraniol one of 3·02. For further identification, to the radioactive samples were added carrier amounts of authentic geraniol and nerol, the 3,5-dinitrobenzoates were prepared, m.p. 60–61° and 35–36°, respectively (reported m.p. 59·5–61·0° and 34·5–35·5°),[21] and the esters were crystallized to constant specific activity without loss of label. Furthermore, the radioactive samples when submitted to GLC with effluent counting showed radioactive peaks at the expected retention times. From this latter measurement no geraniol was detected in the nerol and vice versa.

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Incubations

Two hundred pea-seeds (Burpee's Blue Bantum treated with chloranil to inhibit microbial growth) were completely immersed in water for 24 hr at about 25°. The partially germinated seeds were macerated in a Waring blender for 70 sec with 150 ml of ice-cold 0·1 M phosphate buffer (pH 7·4) which was 0·45 M in sucrose and 0·01 M in $MgSO_4$. The resulting slurry was strained through cheese-cloth and used immediately without centrifugation. 20 ml of the homogenate, 13 mg of ATP, 3·5 mg of NADPH, and 12 mg of nonradioactive MVA (DBED salt) were flushed thoroughly with nitrogen, and geraniol-1-^{3}H ($6{\cdot}95 \times 10^7$ dpm equivalent to 0·34 mg) was added as a solution in 1·0 ml of acetone. After reflushing with nitrogen, the mixture was sealed and incubated for 24 hr at 25°. The homogenate was extracted four times with 75-ml portions of ether.

In the incubations from which squalene was isolated, the ether extract was evaporated to dryness and 25 ml of 5% ethanolic KOH added. After 18 hr 100 ml of water were added and the mixture extracted three times with 25-ml portions of ether, and the ether layer was washed with water and dried (Na_2SO_4). The ether was evaporated to a small volume and chromatographed on 5 g of 2 per cent deactivated alumina.

In the incubations from which geraniol pyrophosphate was isolated the residue from the first extraction of the aqueous homogenate was extracted with three 20-ml portions of collidine, the precipitated protein and salts being discarded. The pyrophosphates were transferred to water by partitioning the collidine against 250 ml of ether and 70 ml of 0·01 M aqueous $NaHCO_3$. The collidine–ether layer was discarded. Residual collidine was removed by extraction of the aqueous layer three times with 100-ml portions of ether. Dissolved ether was removed by rotary evaporation at room temperature for 30 min. There remained 71 ml of aqueous solution of pyrophosphate.

From an incubation of geraniol-1-^{3}H ($9{\cdot}3 \times 10^7$ dpm) $1{\cdot}6 \times 10^6$ dpm was obtained in the hydrocarbon fraction of the alumina chromatogram. This was dissolved in 25 ml of acetone and HCl passed through the solution at 0° for 30 min. Authentic squalene hexahydrochloride (m.p. 109–111°, 20·5 mg) was then added and crystallized from acetone and from methanol–water to give the results of Table 1.

From the incubation of geraniol-1-^{3}H ($6{\cdot}9 \times 10^7$ dpm) $1{\cdot}3 \times 10^6$ dpm was obtained in the collidine fraction. After the radioactive metabolite was transferred to an aqueous solution and washed with ether, an aliquot (5/71) which contained $9{\cdot}2 \times 10^4$ dpm was extracted again with ether. The ether contained only $1{\cdot}3 \times 10^3$ dpm or 1·4% of the radioactivity which represents the maximum amount of nonesterified geraniol present. The aqueous layer of a similar aliquot (5 ml) was adjusted to pH 9 with glycine buffer and 20 mg (100 Bodansky units/mg) of calf intestine alkaline phosphatase (Mann Research Laboratories) and 2 drops of 0·05 N $MgCl_2$ solution added. After 18 hr at room temperature the solution was extracted with ether yielding $9{\cdot}3 \times 10^4$ dpm. The enzymatic hydrolysis procedure has been described by Cramer *et al.*[22] The ether-extractable product was submitted to gas–liquid chromatography as shown in Fig. 1.

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